PROTEINS ASSOCIATED WITH CAULIFLOWER MOSAIC VIRUS INFECTION AND THEIR INTRACELLULAR LOCATION

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CHAPTER I

INTRODUCTION

Cauliflower mosaic virus (CaMV) is one of the Caulimoviruses, a group of plant viruses which have double-stranded DNA as their genetic material. There are at least five members of the Caulimovirus group, of which CaMV has been the most thoroughly studied. Since the recent advent of genetic engineering technology, these DNA plant viruses have assumed increased importance. As the only known double-stranded DNA plant viruses they are being considered as one of the most promising potential vectors for genetic manipulation in plants. Thus, it has become increasingly important to obtain a deeper knowledge of the biology and chemistry of these viruses.

CaMV has a host-range limited almost entirely to members of the Cruciferae family. For use in recombinant DNA work it is essential that a virus not be transmissable by aphids. CM4-184 is an isolate of CaMV that is not aphid transmissable and is therefore of particular interest.

All Caulimoviruses produce characteristic inclusion bodies in the cytoplasm of the host. Although considerable knowledge has been gained concerning the nucleic acid, somewhat less is known about the virus-associated proteins. Electron microscopic information on the size, shape, location and basic composition of inclusion bodies is available. However, the molecular weights, amino acid compositions,

and number of proteins that are associated with CaMV inclusion bodies are not known. Nothing is known about the biological significance of any of these proteins.

The purpose of this work was to increase our knowledge of the proteins associated with CaMV and its inclusion bodies.

CHAPTER II

LITERATURE REVIEW

General

The Caulimovirus group of plant viruses includes: Cauliflower Mosaic Virus (CaMV), Dahlia Mosaic Virus (DaMV), Carnation etched ring virus (CERV), Mirabilis mosaic virus and Strawberry vein banding virus. The main characteristic that groups these viruses together is the presence of DNA as their genetic material. They do, however, have other basic characteristics in common: size and shape; localization in a particular type of inclusion; and some common serological relationships (Shepherd, 1977).

Each member of the group has a specific, limited host range with very little overlap between the members. CaMV's host range is limited to members of the Cruciferae family in nature (Broadbent, 1957) and is transmissable experimentally only to Nicotiana clevelandii (Hills and Campbell, 1968) and Datura stramonium (Lung and Pirone, 1973).

CaMV causes a mosaic-type of disease on infected plants. Vein clearing (loss of chlorophyll along veins), vein banding (green bands of tissue along veins), and leaf chlorotic mottling occur (Shukla and Schmelzer, 1972). Severe crinkling of systemically infected leaves occurs (Shukla and Schmelzer, 1972).

Tissue infected with CaMV, and most other virus-infected plant

tissues, contain inclusion bodies, the microscopic appearance of which is distinctive for each virus group (Christie and Edwardson, 1977). With most viruses these inclusion bodies can be found in many different parts of the cell. However, in the case of CaMV they are found exclusively in the cytoplasm (Shepherd, 1977). Their occurence in the nucleus has been reported (Fujisawa et. al., 1967) but subsequent EM studies did not substantiate that report (Rubio-Huertos et. al., 1968; Christie and Edwardson, 1977). CaMV inclusion bodies apparently consist of a protein matrix in which both empty and full virions may be present (Martelli and Castellano, 1971).

There are two types of aphid-transmitted viruses: persistent (those which are ingested and are subsequently excreted from the salivary glands) and non-persistent (those which do not move internally being carried on the stylet). CaMV was first thought to be a typical non-persistent virus, but subsequent work showed it to be atypical in that it remains infective much longer than a normal non-persistent virus. Day and Venables (1961) showed that Brevicoryne brassicae could retain infectivity for up to three days although retention never followed a molt in which the stylet is shed along with the exoskeleton. Injection of hemolymph from an aphid fed on infected plant material into the hemolymph of an aphid that had not been fed on infected material did not result in transmission of the virus, indicating that the virus may not move internally.

These results indicated that CaMV is probably transmitted in a non-persistent manner. Recently, Lung and Pirone (1973, 1974) have reported the requirement of an acquisition factor for aphid transmissability. They have shown that when normally non-transmissable

isolates of CaMV are probed by an aphid that has first probed a plant which is infected with a transmissable isolate, the aphid is able to then transmit the non-transmissable isolate. This indicates that an acquisition factor is present in infected tissue that somehow aids in the transmission of CaMV.

CaMV Nucleic Acids

At the present time more is known about the nucleic acid of CaMV than about any other component of the virus. This was the first plant virus reported to contain DNA as its genetic material (Shepherd et. al., 1968). Since then several other plant viruses have been reported to contain DNA and have thus been designated as Caulimoviruses. The DNA of CaMV was shown to be double-stranded (Shepherd et. al., 1970).

The DNA has a bouyant density of 1.702 g/ml in cesium chloride density equilibrium centrifugation (Shepherd et. al., 1970), which along with other data suggests a GC content of about 43%. It has a molecular weight of approximately 4.4-4.7 x 10^{-6} (Shepherd and Wakeman, 1970; Russell et. al., 1971), and exists in both linear and circular forms when isolated although only the circular form is infective (Hull and Shepherd, 1975). The circular molecule has some single-stranded regions as indicated by sensitivity to S1 nuclease (Hull and Shepherd, 1977). There is also some apparently randomly dispersed RNA present (Hull and Shepherd, 1977).

Restriction maps of several of the isolates have been constructed (Meagher et. al., 1977; Hull and Howell, 1978; Lebeurier et. al., 1978; Gardner, unpublished data). Isolate CM4-184 which lacks aphid-transmissability has a 450 base pair deletion relative to CaB

indicated in the restriction map shown (Figure 1).

CaMV Proteins

In a 1970 issue of C.M.I./A.A.B. Description of Plant Viruses report, Shepherd indicated that there was no information available on the proteins associated with CaMV. In the years since 1970 there have been a number of reports concerning the structural proteins of the virus (Tezuka and Taniguchi, 1972; Kelly et. al. 1974; Brunt et. al. 1975; Hull and Shepherd, 1976; Al Ani et. al. 1979). There has been considerable disagreement as to the number of proteins and as to which proteins are the major structural proteins and which ones are degradation products.

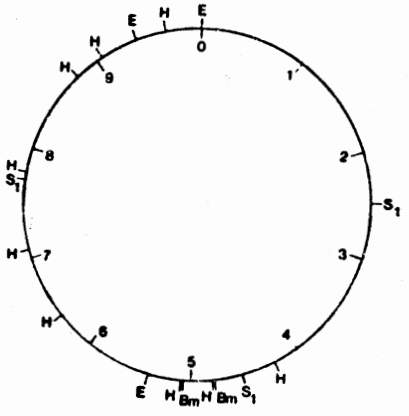
Tezuka and Taniguchi (1972) detected only two proteins upon analysis of the purified virus by SDS-PAGE. The major and minor proteins had molecular weights of 33,000 and 68,000 respectively. Kelly et. al. (1974) detected at least six proteins with approximate M.W.'s of 100, 92, 67, 40, 32 and 27,000. The 32,000 dalton protein made up 69% of the viral protein with the 67,000 dalton protein making up another 18% of the total protein. The two highest M.W. proteins stained faintly with Schiff's reagent and thus may be glycoproteins.

Following this report, Brunt et. al. (1975) reported the presence of three proteins that they considered to be the major structural proteins and the presence of several other peptides that, due to their appearance or disappearance in aged virus preparations, were considered to be degradation products or aggregates of the three major proteins. Their major protein had a M.W. of approximately 42,000 with two minor

Figure 1. CaMV DNA Restriction Maps. Enzymes used: E(EcoRI), H(Hind III), S_1(S_1 nuclease), Bg(Bgl II), Bm(Bam I). (a) CaB Davis, (b) CM4-184, (c) CM4-184 showing deletion.

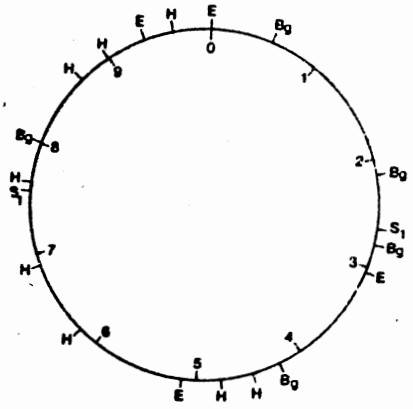
a.

Cabbage B



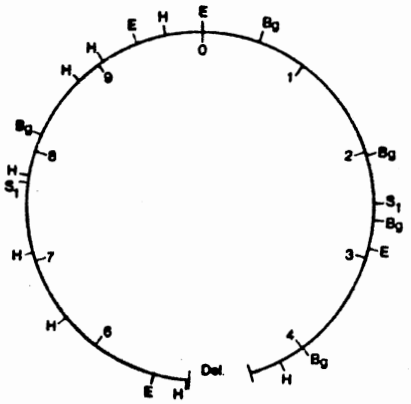
b.

CM4-184



c.

CM4-184



components of 68,000 and 55,000. They also carried out an amino acid analysis of the complete virus particles which indicated a high content of the basic amino acids lysine (18%) and arginine (5%). The distribution of these amino acids among the different polypeptides has not yet been determined and the highly basic nature of the proteins suggests that they may function similarly to histones although this has not been proven. No bands stained with Schiff's reagent. They considered the high M.W. bands to be aggregates of the low M.W. polypeptides.

In contrast, Hull and Shepherd (1976) found four major polypeptides and two that occurred occasionally. One of the latter was thought to be a degradation product of the major polypeptide. The M.W.'s of the four major polypeptides and their molar ratios were 37,000 (0.77), 64,000 (0.15) and 96,000 and 88,000 (sum of the last two: 0.08). They reported faint Schiff staining of the higher M.W. bands and light staining of the lower M.W. bands, but concluded that confirmation of glycosylation of these peptides would have to await further tests as the periodate-Schiff test is not proof of glycopeptides. No phosphorylated polypeptides were found when CaMV grown in the presence of ^{32}P was subjected to SDS-PAGE (Hull and Shepherd, 1976).

Al Ani et. al. (1979) attempted to clarify the confusion that the above four reports caused. Seven polypeptides designated P1 through P7 were found with M.W.'s as follows: P1--33,000; P2--37,000; P3---39,000; P4--42,000; P5--49,000; P6--55,000; P7--70,000-85,000. They indicated that the method of purification of the virus can result in different patterns of relative intensities of the different

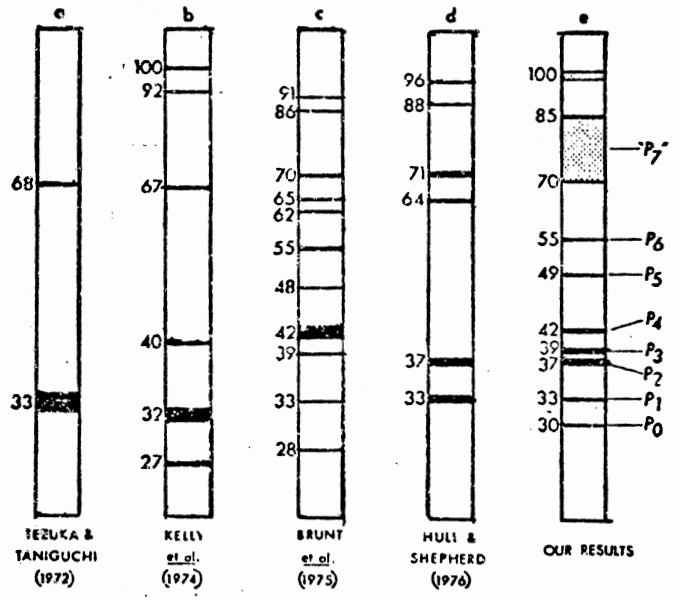
bands due to degradation of the major polypeptide and aggregation of these degradation products. They carried out one-dimensional peptide mapping using the technique developed by Cleveland et. al.,(1977). The results indicated that the major coat protein of CaMV is the 42,000 dalton protein (P4). Degradation of this protein during purification resulted in the production of P1, P2, P3, and P7. The report also indicated that P5 (49,000) and P6 (55,000) were present as minor constituents and had sequence homologies with P4. From this data Al Ani et. al. have suggested that the virus has a simple structure with only one major protein - P4 - and small amounts of P5 and P6 present. It is apparent that it is quite possible to obtain different results depending on the method of purification.

Figure 2 shows diagrammatically the comparisons of the polypeptides from all of these reports.

Inclusion Bodies

Martelli and Russo (1977) described inclusion bodies simply as intracellular structures produced de novo as a result of viral infections. The first report of plant cell structures associated with viral infections was made by Iwanoski (1903) while studying tobacco mosaic virus. Since that time it has become apparent that most plant viruses produce or cause to be produced intracellular structures that have come to be known as inclusion bodies. These inclusion bodies attain a multitude of sizes and shapes that appear to be governed by virus-host interactions or virus-virus interactions. Shape is often a result of the geometrical restrictions within the host cell.

Figure 2. CaMV Protein Comparisons



A comparison of the polypeptide profiles described for CaMV by: (a) Tezuka and Taniguchi (1972b); (b) Kelly *et al.* (1974); (c) Brunt *et al.* (1975); (d) Huli and Shepherd (1976); (e) our results. Band intensities are only approximations deduced from densitograms or eye estimation of band patterns. Band thickness is proportional to relative abundance in each virus preparation. Band positions were adjusted by plotting log molecular weight vs migration. Source: Al Ani et. al. (1979)

The composition of inclusion bodies is also extremely variable and complex (Martelli and Russo, 1977). They may consist of virus components only, host components only, or virus and host components together. They also have been found in most compartments of a plant cell. For that reason, inclusion bodies are often grouped according to their intracellular location. They are either nuclear or cytoplasmic. They are grouped into one of two basic groups based on their gross morphology: (1) crystalline, which are generally composed of pure or at least nearly pure aggregates of virions and (2) amorphous, which is any non-crystalline aggregate of materials - whatever their origin - arising in the cytoplasm as a result of viral infections (Martelli and Russo, 1977).

CaMV inclusion bodies fall into the amorphous category. The first person to view inclusion bodies in the Caulimoviruses was Goldstein (1927) and he game them the name "X-bodies". They have since become known simply as inclusion bodies with "X-bodies" being reserved for the TMV inclusions for which the term was first coined. The different members of the Caulimovirus group all contain inclusion bodies that appear to be of the same general type although they differ somewhat in size and shape.

CaMV inclusions have been studied quite intensively both by light microscopy and by electron microscopy. When being studied by light microscopy the inclusions have usually been stained with 0.5-1% phloxine (a protein stain) (Fujisawa, 1967) or could be seen well using interference contrast optics (Mamula and Milicic, 1968). Recently the use of Azure A (an RNA stain) as a stain for light microscopy has been introduced by Christie and Edwardson (1977).

CaMV inclusions are found predominantly in the cytoplasm although there have been reports of inclusions within the nucleus (Fujisawa, 1967). These reports are based on light microscope work and have not been substantiated using electron microscopy. The inclusions do not possess an external membrane.

The vacuolated-conglomerative inclusions have vacuole-like areas randomly distributed throughout the matrix. The significance of these vacuoles has not been deciphered yet, although it has been suggested that there may be a network of lacunae throughout the inclusion body. Virions are usually found in these vacuoles, but are also found embedded throughout the matrix of the inclusion bodies. The non-vacuolated inclusion bodies have no visible virions associated with them though they do have viral DNA associated with them (Shepherd and Wakeman, 1977).

This matrix material appears to be mainly protein as shown by enzyme-digestion tests (Martelli and Castellano, 1971). RNA and DNA are also present as indicated by staining reactions (Martelli and Castellano, 1971). There is debate as to the presence of full virions in the inclusion bodies due to the fact that time of exposure to stain reagents and pH will affect the penetration of these stains which could give the appearance of the presence of full virions that may be empty. Following DNase digestion, however, all virions appear to be empty, so it is likely that both full and empty virions occur in the inclusion bodies as the removal of DNA from the core of full virions gives them an empty appearance (Conti et. al., 1972).

When infected leaf tissue slices are observed by EM there are usually large numbers of ribosomes associated with the inclusion

bodies (Petzold, 1968). This would seem to indicate that protein synthesis is occuring there and lends credence to the thought that the inclusion bodies are the site of viral protein synthesis and assembly.

Although much microscopy has been carried out on the inclusion bodies, there has been only one preliminary report on the matrix protein (Shepherd, 1977). McWhorter (1965, p. 287) stated in his review of plant virus inclusions that "The current lack of interest by plant virologists in inclusions is appalling. . .". Since that time there has been considerably greater interest in them, however with regard to the biochemistry of the inclusion bodies there is still a long way to go.

CHAPTER III

MATERIALS AND METHODS

Preparation of Infected Tissue

CaMV isolates CM4-184, CaB Davis and NY 8153 were obtained from R. J. Shepherd (U.C., Davis, CA.). These isolates were diluted to a concentration of two µg/ml in a 1% K_2HPO_4 buffer for inoculation of either turnip (Brassica rapa, cv. Just Right) or mustard (Brassica perviridis, cv. Tendergreen). Following application of a small amount of carborundum to the fourth and fifth leaves of the plants, a cotton Q-tip was dipped into the inoculum and rubbed onto the carborundum-treated leaves. The plants were grown in a controlled growth chamber at a temperature of 18.3°C at night and 21.7°C during the day with a 24 hour cycle of 12 hr. night/12 hr. day. Five to six and a half weeks later the leaves were harvested for virus isolation. After harvest, the plants infected with CM4-184 were allowed to grow new leaves and these contained virus. These new leaves were used for inclusion body preparations. However, if infected with either NY 8153 or CaB Davis, new leaves did not appear because of the virulence of these strains, thus original leaves had to be used for inclusion body preparations.

Virus Preparation

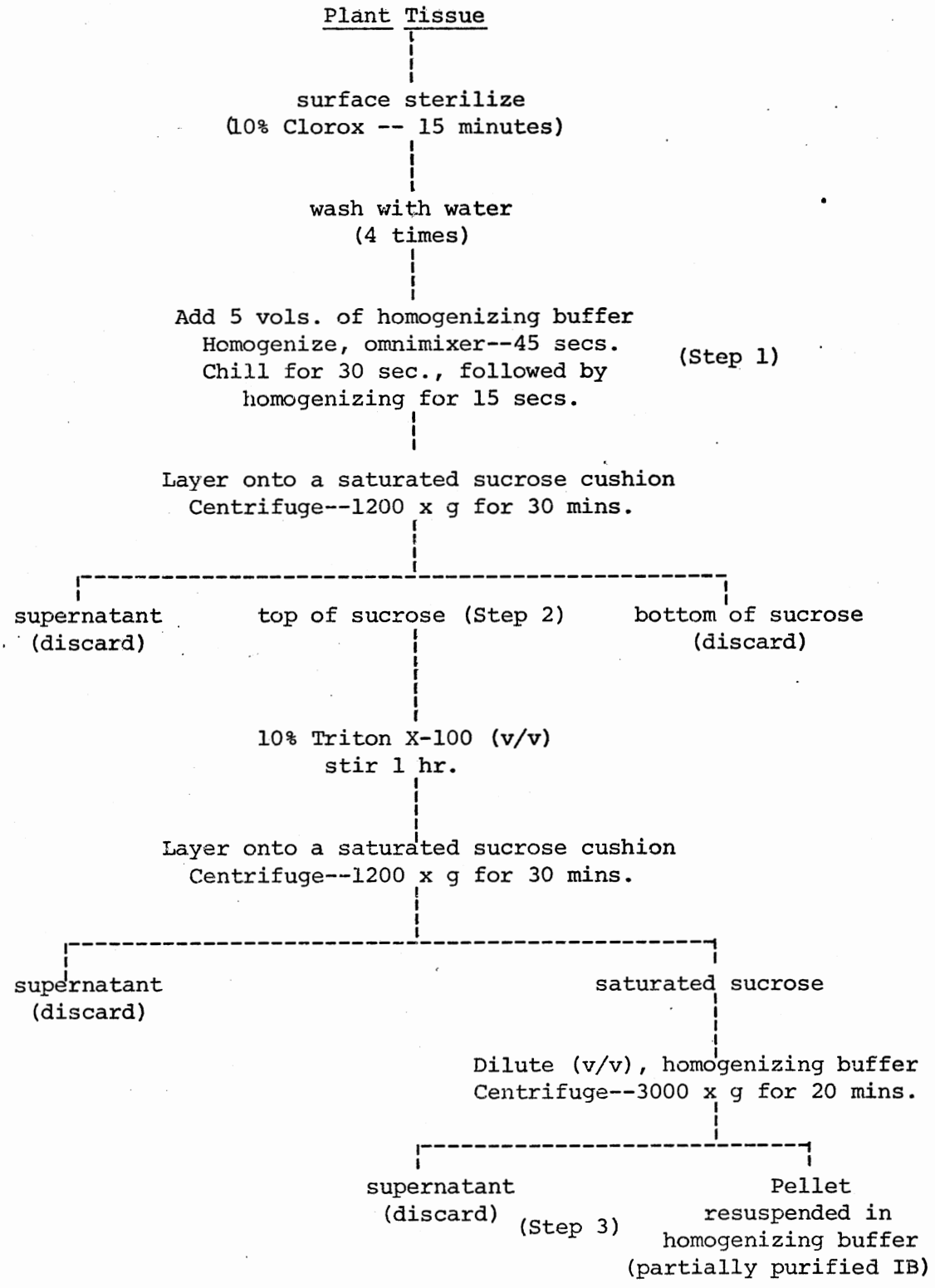
Virus was purified from infected plants by the procedure of Hull

and Shepherd (1975). In one instance a procedure developed by Al Ani et. al. (1979) was used. This procedure involves first freezing the harvested plant tissue immediately following harvest and then thawing prior to purification by the Hull and Shepherd (1975) procedure. This freeze-thaw step is reported to result in less degradation of the viral coat proteins.

Inclusion Body Purification

Inclusion bodies were partially purified using a series of centrifugations into saturated sucrose cushions as shown in the purification scheme (Figure 3) using a modification of a procedure developed by Shepherd (personal communications). All procedures were carried out at cold room temperatures (4°C). The plant tissue was first surface sterilized in a solution of 10% Clorox for fifteen minutes followed by four washes with distilled water. The tissue was then cut into small pieces and placed in five volumes of a buffer (50 mM Tris-HCl, 10 mM Na_2SO_3, 8.5% sucrose, 10 mM MgCl, 25 mM KCl, pH 7.3). It was then homogenized in an omnimixer for 45 sec., chilled for 30 sec. and then further homogenized for 15 sec. The homogenized material was strained through four layers of cheesecloth and then layered onto a 15 ml saturated sucrose cushion (approximately 15 mls of extract per tube). These tubes were then centrifuged in a Sorvall GLC-2 table top centrifuge in a HL-4 rotor at 1200 xg for 30 min. The supernatant was removed using a pasteur pipette and discarded. The top of the sucrose cushion containing the chloroplast band was removed with a pasteur pipette and mixed with an equal volume of 10% Triton X-100 to give a final concentration of 5%. This

Figure 3. Purification of Inclusion Bodies


Plant Tissue
surface sterilize
(10% Clorox -- 15 minutes)
wash with water
(4 times)
Add 5 vols. of homogenizing buffer
Homogenize, omnimixer--45 secs.
Chill for 30 sec., followed by
homogenizing for 15 secs.
(Step 1)
Layer onto a saturated sucrose cushion
Centrifuge--1200 x g for 30 mins.
supernatant
(discard)
top of sucrose (Step 2)
bottom of sucrose
(discard)
10% Triton X-100 (v/v)
stir 1 hr.
Layer onto a saturated sucrose cushion
Centrifuge--1200 x g for 30 mins.
supernatant
(discard)
saturated sucrose
Dilute (v/v), homogenizing buffer
Centrifuge--3000 x g for 20 mins.
supernatant
(discard)
(Step 3)
Pellet
resuspended in
homogenizing buffer
(partially purified IB)

mixture was then stirred at 4°C for one hour.

The mixture was then layered onto another saturated sucrose cushion and centrifuged at 1200 x g for 30 minutes in the GLC-2 centrifuge. The supernatant was once again removed and discarded and the remaining sucrose cushion was diluted with one volume of the homogenizing buffer. A pelleting run was then carried out at 3000 x g for 20 minutes in a Sorvall RC-2B centrifuge. These partially purified inclusion body pellets were then resuspended in a small amount of the homogenizing buffer and used for either SDS-PAGE or other experiments.

SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out using the procedure developed by Laemmli (1970). The 10% separating gels contained 10 ml of 30% acrylamide/0.8% BIS, 15 ml of a buffer (pH 8.8, 0.37 M Tris-HCl, 0.4% SDS), 5 ml of water, 100 µl of ammonium persulfate (APS), and 10 µl of TEMED (tetramethylethylene diamine). The gel was poured into a slab gel mold and about five ml of isobutanol were layered onto the gel material. Once the separating gel had polymerized the isobutanol was removed and a 3.5% stacking gel was poured onto the separating gel. The stacking gel consisted of 1.5 ml of the 30% acrylamide/0.8% BIS solution, 6 ml of a buffer (pH 6.8, 0.5M Tris-HCl, 0.4% SDS), 4.5 ml of water, 40 µl of APS and 10 µl of TEMED. In cases where the separating gel was 15% acrylamide instead of 10%, 15 ml of 30% acrylamide/0.8% BIS was added instead of 10 ml and no water was added.

Once the stacking gel was polymerized (usually two hours or more), the gel was placed in a slab gel running apparatus. A running buffer

(0.19 M glycine, 25 mM Tris, 0.1% SDS, pH 8.3) was added to the lower buffer chamber and all air bubbles were cleared from the bottom of the gel using a syringe with a bent needle. Any remaining liquid in the sample wells was removed using pieces of filter paper. Samples were then applied to the sample wells followed by layering of running buffer onto the samples. Once sufficient buffer had been layered to ensure no spillover from one well to another, the top buffer chamber was filled with the running buffer.

The gels were then electrophoresed at three mamp overnight (approx. 18 hrs.), and then at 15 mamps until the BPB marker reached the bottom of the gel at which time the gel was removed for staining. The gels were placed in a 25% isopropanol/10% acetic acid solution for about one hour to remove the SDS. They were then stained for one to two hours with 0.1% Coomassie Blue G stain dissolved in 25% isopropanol/ 10% acetic acid. Destaining was carried out in 10% acetic acid and required at least 24 hours for complete destaining.

Sample Preparation for SDS-PAGE

Samples were prepared for SDS-PAGE by dissolving them in an appropriate amount of a dissolving buffer: 5% SDS (sodium dodecyl-sulfate), 5% 2-mercaptoethanol, 5M urea, 0.05M Tris-HCl and 25 µl of Bromophenol Blue (BPB) as a tracking dye. The samples were heated in a boiling water bath for two minutes and then applied to the sample wells.

Molecular Weight Markers

The M.W. markers were obtained from Sigma Chemical Company and were dissolved in the same buffer that the samples were dissolved in.

The SDS-6 M.W. marker kit was used. The proteins present in the kit and their molecular weights were: (1) BSA - 66,000; (2) Ovalbumin - 43,000; (3) Pepsin - 34,700; (4) Trypsinogin - 24,000; (5) β-lactoglobulin - 18,400; and (6) Lysozyme - 14,300.

Inclusion Body Staining

Two different techniques were utilized for staining the inclusion bodies although the Azure A method was used exclusively in later experiments. For staining of epidermal strips of leaf tissue, a piece of epidermal tissue from the bottom side of a leaf, preferably along a vein, was first removed. This epidermal strip was allowed to float, inner side down, on a small amount of the stain solution and then rinsed for 30-60 sec. in the appropriate solution. It was then placed in a drop of the appropriate solution on a microscope slide and a coverslip applied.

When staining with Phloxine B, the stain was at a concentration of 0.1% in a 0.85% NaCl solution. The strip was rinsed with water and then placed on the slide in a drop of water. When observing extracted material in Phloxine B stain, a drop of the extract was placed directly on the slide along with a drop of the stain and observed immediately.

When staining with Azure A (Christie and Edwardson, 1977), two solutions were made: (1) 0.1% Azure A in 2-methoxyethanol, (2) 0.2M disodium phosphate in distilled water. Just prior to staining, 18 parts of the dye were mixed with two parts of the 0.2M Na_2HPO_4. Epidermal strips were floated on this solution for 15-30 minutes. They were then rinsed for 15-30 seconds in absolute ethanol, the strip was placed in a drop of immersion oil on a glass slide and viewed

immediately. When staining extracts, one drop of extract was placed on a slide along with a drop of the mixed Azure A stain and viewed immediately. None of these techniques were appropriate for making permanent slides. The Azure A technique gave the best results.

Amino Acid Analysis

To obtain the protein for amino acid analysis a slab gel containing five lanes of infected material and five lanes of uninfected control material was run in SDS-PAGE as indicated above. The gels were stained as described above. The area of the gel containing the 61,000 dalton protein was cut out of the gel taking care not to contaminate the material with proteins or amino acids present on the experimenter's hands. The gel slice was weighed and placed in two µl of glacial acetic acid/mg of gel and the protein was extracted for approximately 10 hours. The gel slice was extracted twice more in 66% acetic acid for periods of 24 hours. The acetic acid extracts were combined and the acetic acid removed by vacuum desiccation over NaOH crystals. The protein was then hydrolyzed in 200 µl of 6N HCl, one µl of thioglycolic acid, 10 µl of 5% phenol *in vacuo* at 100°C for 24 hours. The acid was removed by vacuum desiccation over NaOH and the remaining amino acids were dissolved in diluting buffer. 100 µl of the sample was loaded onto an automatic amino acid analyzer. Corresponding gel slices from the lanes with uninfected material were also analyzed in the same manner to act as controls.

EM Procedure

Samples were prepared for EM according to the inclusion body

procedure above. At step three of the procedure the pellets were fixed immediately in buffered glutaraldehyde for two hours. They were then washed two-three times in water and then post-fixed in 2% OsO_4 for one hour, followed by en bloc staining overnight in aqueous 2.5% uranyl acetate at 60°C. The samples were dehydrated with increasing concentrations of ethanol followed by washing with propylene oxide. They were then embedded in DER and placed in a vacuum oven for approximately 48 hours. The samples were thin-sectioned and then viewed using transmission electron microscopy.

CHAPTER IV

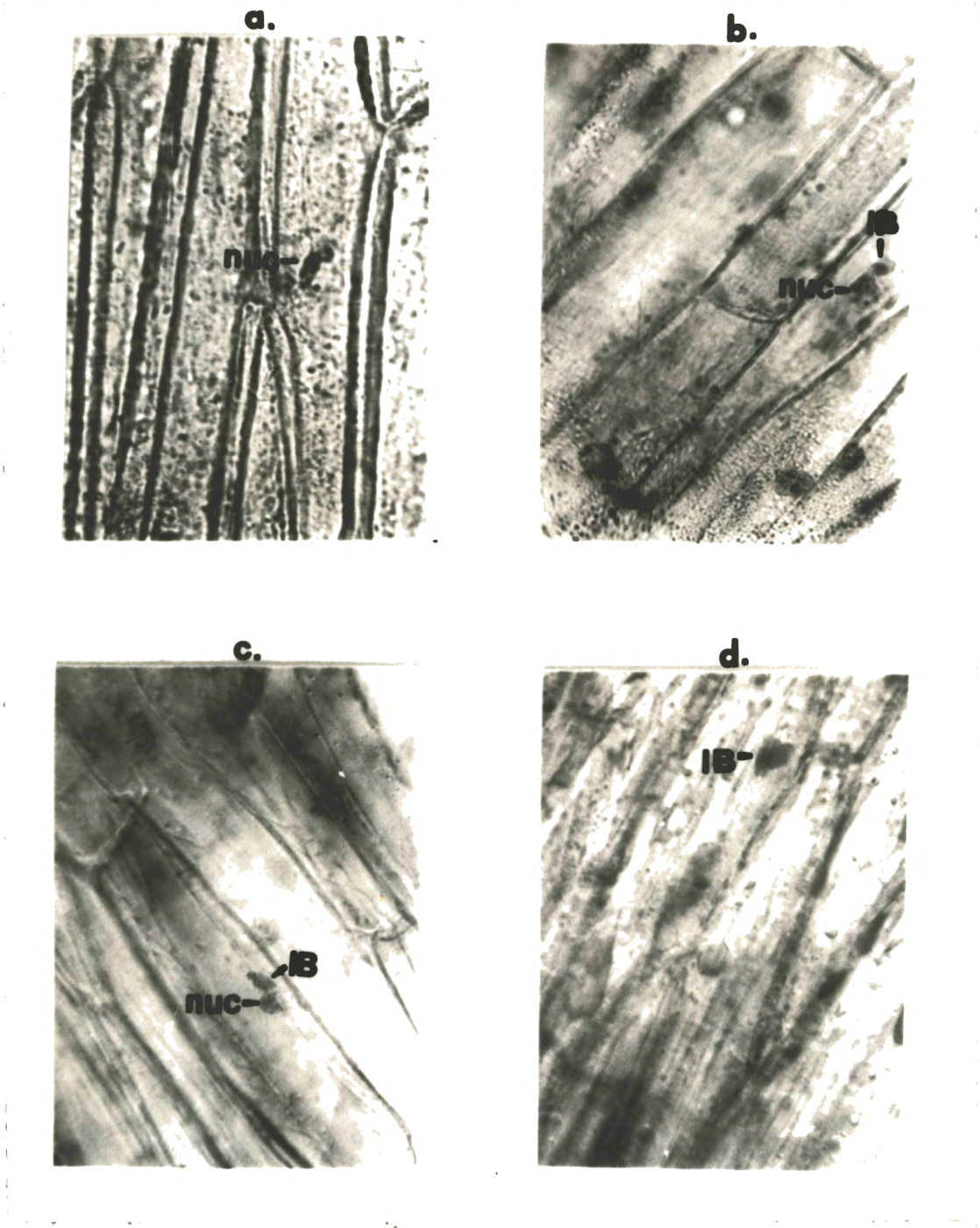
RESULTS

Purification of Inclusion Bodies

In order to purify inclusion bodies a method had to be found to identify them during the purification. Because of the characteristic appearance of the bodies in the light microscope after staining, light microscopy was used. The first experiments utilized Phloxine B. With this stain, once the plant material had been homogenized, it became difficult to differentiate between cell organelles and inclusion bodies. The Azure A technique developed by Christie and Edwardson (1977), gave much clearer results. With this method inclusion bodies are purplish; nuclei are very light blue; and nucleoli are very dark blue. The only other material that stained appreciably in leaf strips was meristematic tissue. The procedure developed allowed the detection of inclusion bodies in homogenates, thus providing a tool to follow purification. The purification of inclusion bodies was also followed by SDS-PAGE of proteins at each step of the purification.

Figure 4 shows the characteristic appearance of inclusion bodies in epidermal strips of infected leaves stained with Azure A. The nucleus with several dark staining nucleoli can be seen in an epidermal strip of an uninfected turnip (Figure 4a). In infected mustard epidermal strips (Figure 4b and 4c), the inclusion bodies are seen

Figure 4. LM Photographs of Infected and Uninfected Epidermal Strips. (a) Uninfected turnip; (b) and (c) Infected mustard; (d) Infected turnip.


a.
nuc
b.
IB
nuc
c.
IB
nuc
d.
IB

next to the nucleus. An inclusion body and large nucleoli can be seen in an infected turnip epidermal strip (Figure 4d). It was once thought (Fijisawa et. al., 1967) that these large nucleoli could be nuclear inclusion bodies of CaMV, but as stated earlier, inclusion bodies have never been observed in the nucleus by EM.

The presence of inclusion bodies in the total extracts at step one of the purification scheme was first verified by LM. Inclusion bodies were present at a low concentration only in the extract of infected plants. When the polypeptides of total extracts of infected and non-infected tissue were compared by SDS-PAGE, the only difference detected was the presence of a 68,000 dalton band in the extract (Figure 5, lanes 1 and 2) of infected tissue but not of uninfected tissue.

After a low speed centrifugation onto sucrose (step two of the purification), the 68,000 dalton band could no longer be detected (Figure 5, lane 3). The only visible difference correlated with infection was an increased amount of a 61,000 dalton band in the sample of infected leaves (Figure 5, lane 3). In the light microscope, large numbers of chloroplasts were seen in both samples but only the sample from infected tissue had inclusion bodies.

After detergent treatment and recentrifugation into sucrose (step three of the purification) there were some obvious differences (Figure 6) associated with infection. First, there was a significant enrichment of the 61,000 dalton polypeptide and another intense band was found at approximately 43,000 daltons. At least four minor bands could be seen: 56,000, 50,000, 41,000, and 39,000 daltons. Of these only the 41,000 dalton band could be found in the pattern from the

Figure 5. SDS-PAGE of Steps 1 and 2 of Purification.[a] lane 1 - infected, total extract, lane 2 - uninfected, total extract, lane 3 - infected, step 2, lane 4 - infected, step 2, lane 5 - standards. [a]See text for description.

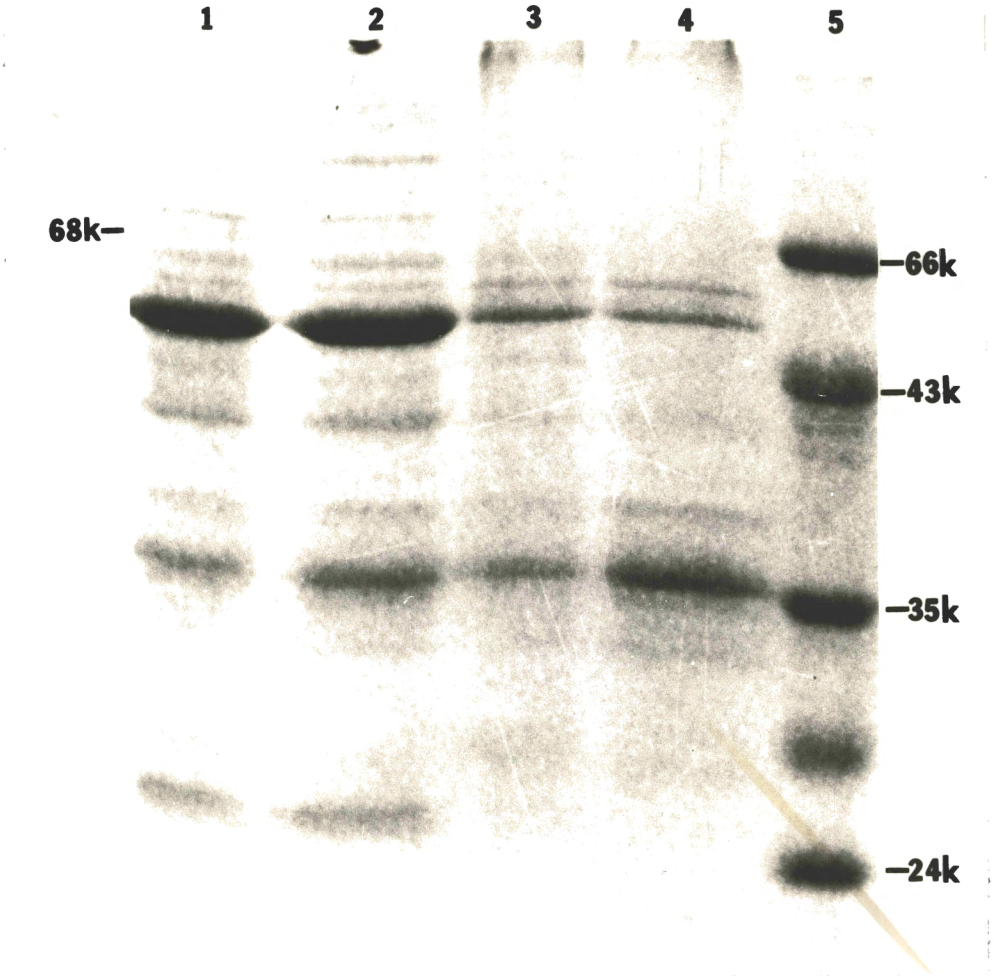
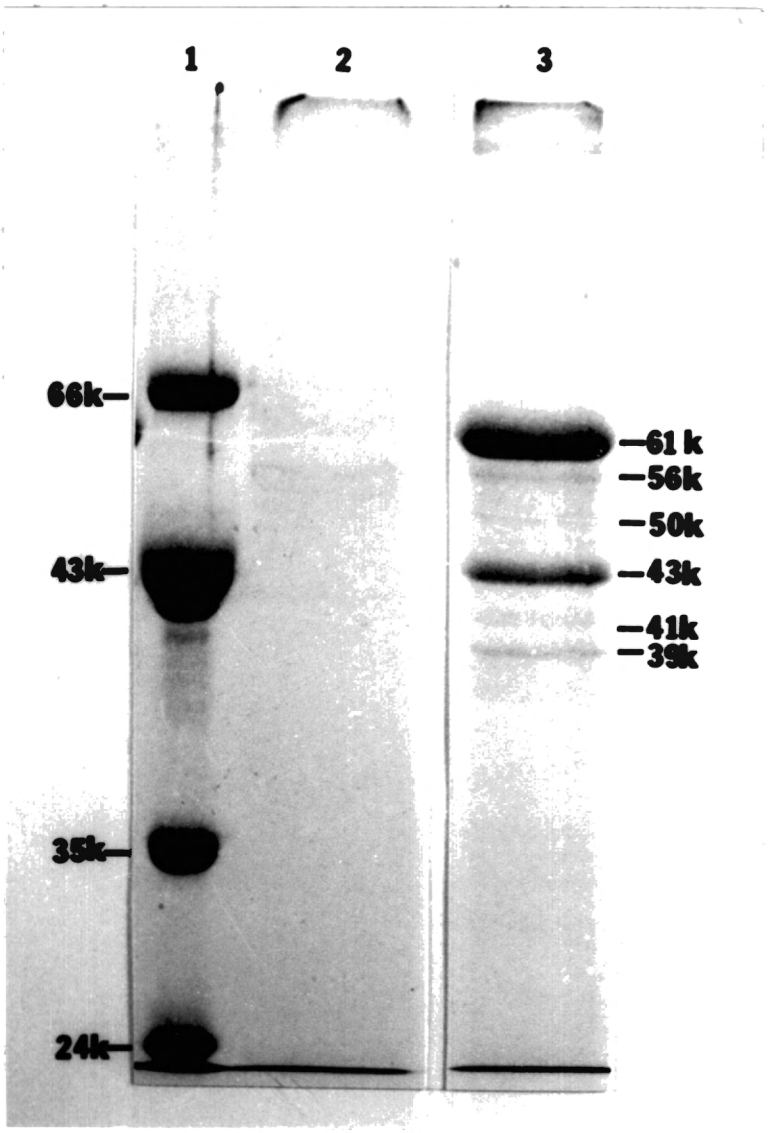

1
2
3
4
5
68k—
—66k
—43k
—35k
—24k

Figure 6. SDS-PAGE of Step 3 of Purification.[a] lane 1 - standards, lane 2 - uninfected, lane 3 - infected. [a]See text for details.


1
2
3
66k
43k
35k
24k
61 k
56k
50k
43k
41k
39k

uninfected leaves. Inclusion bodies could be seen by LM in the sample from the infected leaves (Figure 7a). There was also material that looked like nuclear material with nucleoli. In the sample from uninfected tissue no inclusions were present, but starch granules and what appeared to be nuclear material with nucleoli were seen (Figure 7c).

The presence of inclusion bodies of both the vacuolated-conglomerative type and the nonvacuolated type in these final samples was verified by EM (Figure 8). The vacuolated inclusion bodies are irregular in shape and contain virions. The non-vacuolated inclusion body is less irregular and no virions could be seen.

Both empty and full virions could be seen (Figure 9) although the observation of both empty and full virions may be artifact caused by poor stain penetration (Davison and Francki, 1969).

Amino Acid Analysis

The 61K band from partially purified inclusion bodies was cut out of the gel shown in Figure 10. The band was cut from lanes one through five and combined to increase the amount of protein recovered. The corresponding positions in lanes six through ten were used as controls. The recovered protein was hydrolyzed and the resulting amino acids analyzed (Table I). Since cysteine and tryptophan are destroyed during hydrolysis and since the high amounts of glycine in the control sample did not allow determination of glycine, the molar percentages are overestimates. Nonetheless proline and valine are present in significantly higher mole percentages in the 61K polypeptide than in isolated virus (Brunt et. al., 1974). Lysine and tyrosine are present in significantly lower amounts in the 61K polypeptide.

Figure 7. LM Photographs at Step 3 of Purification. (a) Infected, turnips; (b) infected, mustard; (c) uninfected, turnips.


a.
IB
b.
c.

Figure 8. EM Photograph at Step 3 of Purification. Note the vacuolated-conglomerative inclusion bodies (vac.) and the non-vacuolated type (non-vac.)

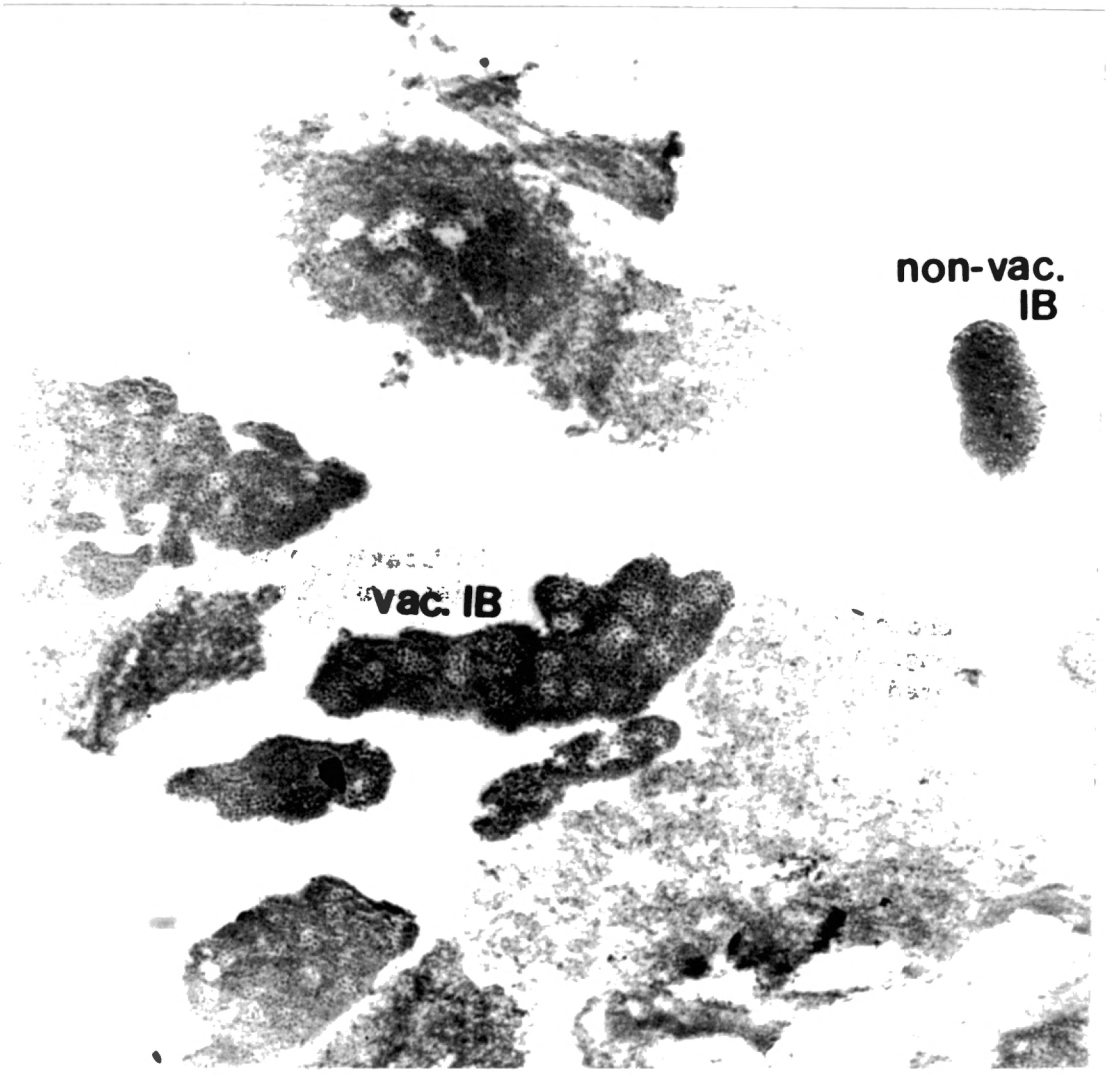

non-vac.
IB
vac. IB

Figure 9. EM Photograph at High Magnification Showing the Presence of "Empty" and "Full" virions in the Vacuolated-conglomerative Inclusion Bodies.

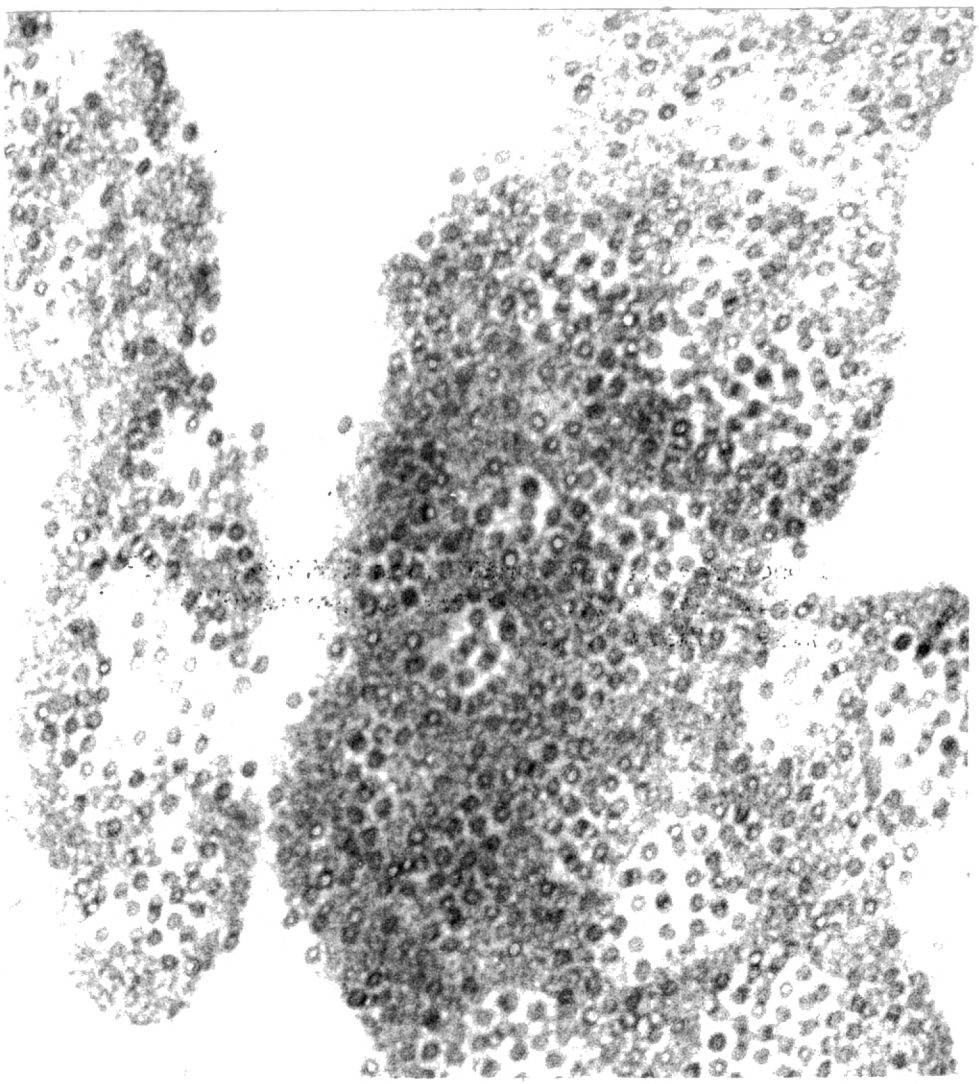


Figure 10. SDS-PAGE at Step 3 of Purification. Lanes 1-5 (infected), lanes 6-10 (uninfected). 61K polypeptide was used for amino acid analysis.

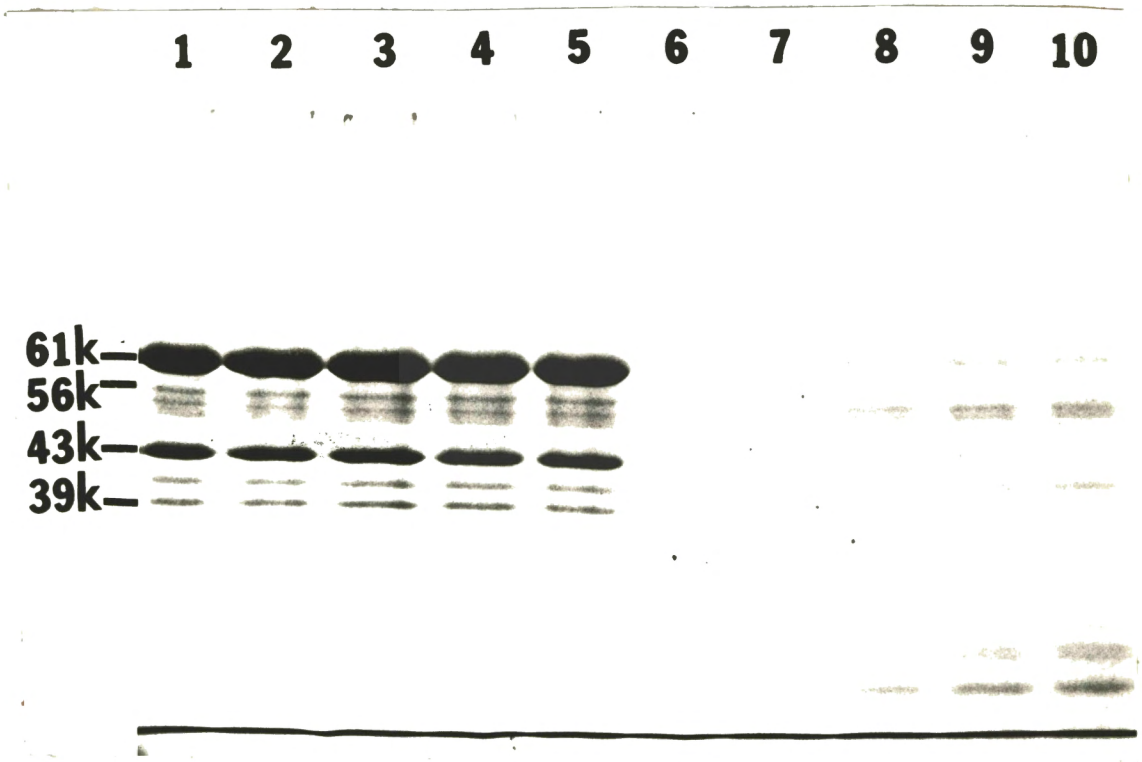

1
2
3
4
5
6
7
8
9
10
61k
56k
43k
39k

TABLE I

AMINO ACID ANALYSIS

Amino Acid	Amino Acid recovered (nmol) 61K band	control	Mole % of 61K band	Brunt et. al. Mole %
Asp	217	68	10.5	8.99
Thr	140	29	7.8	6.57
Ser	136	64	5.1	4.72
Glu	266	83	12.9	11.52
Pro	146	25	8.6	3.46
Gly	182	429*	---	6.91
Ala	155	56	7.0	5.07
Val	125	37	6.2	1.38
Met	42	33	0.6	1.61
Ile	139	37	7.2	6.80
Leu	170	52	8.3	7.49
Tyr	59	27	2.3	5.65
Phe	80	14	4.7	3.11
His	56	25	2.2	1.04
Lys	205	39	11.7	17.97
Arg	97	29	4.8	4.84
Cys	---	--	---	2.88

*This high amount of glycine is probably contamination from the SDS-PAGE buffer.

Amino acid analysis was also carried out on the 56K, 43K, and 39K bands, but the background was too high to obtain significant data.

Strain Comparison

The polypeptides associated with inclusion bodies from plants infected with three isolates (CM4-184, NY 8153, and CaB Davis) of CaMV were compared (Figure 11). The same polypeptides are present in inclusions from all three sources although there are some slight differences in the mobilities of at least two bands. The 39K band and the 56K polypeptides in the CM4-184 sample lane migrate more slowly than corresponding bands in the NY 8153 and CaB Davis samples. Although it cannot be measured on this SDS-PAGE gel, it is possible that the 43K band also migrates more slowly in CM4-184. This would be consistent with the possibility that the 39K band is a degradation product of the 43K polypeptide.

Identification of Proteins Associated with Virus

In an attempt to identify as many proteins associated with viral infection as possible, SDS-PAGE gels were run on several other fractions from the inclusion body purification. When the total extract and the supernatant from step one of the purification scheme were pelleted at 30,000 x g and proteins of the pellet were separated by SDS-PAGE, no infection associated proteins were identified (Figure 12). SDS-PAGE patterns on the TCA precipitates of the supernatants from the 30,000 x g centrifugations show the presence of one high M.W. polypeptide in the neighborhood of 100,000 in all three infected samples (Figure 13). The only other observable difference in this

Figure 11. Strain Comparison of Inclusion Body Fraction Polypeptides by SDS-PAGE. Lane 1 - standards; Lane 2 - CaB Davis; Lane 3 - NY 8153; Lane 4 - CM4-184; Lane 5 - uninfected.

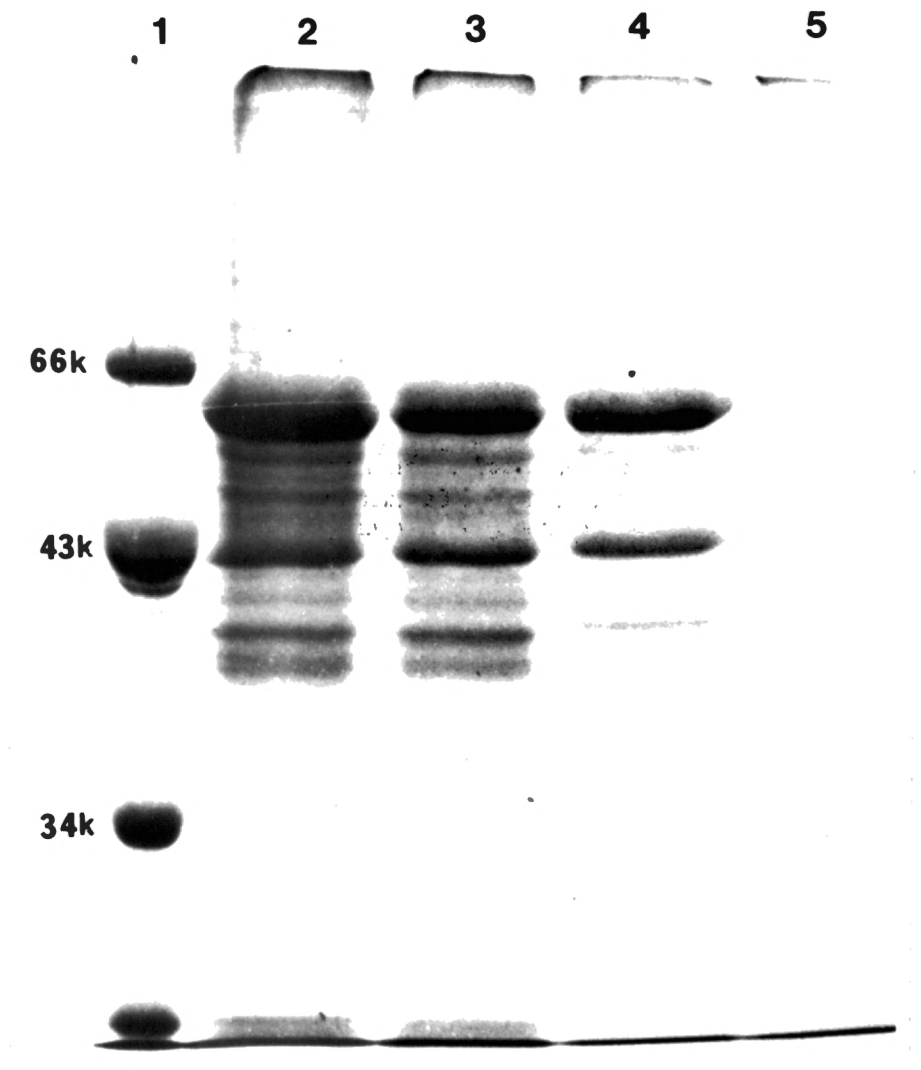

1
2
3
4
5
66k
43k
34k

Figure 12. Strain Compairson of 30,000 x g Pellets by SDS-PAGE. Lane 1 - CaB Davis; Lane 2 - NY 8153; Lane 3 - CM4-184; Lane 4 - uninfected; Lane 5 - standards.

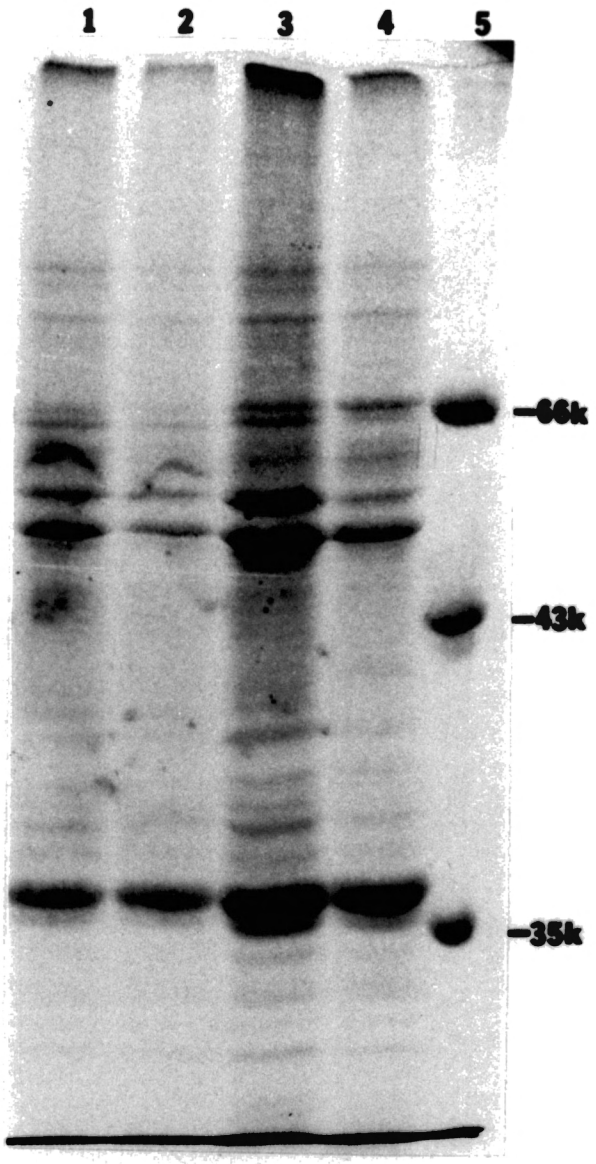
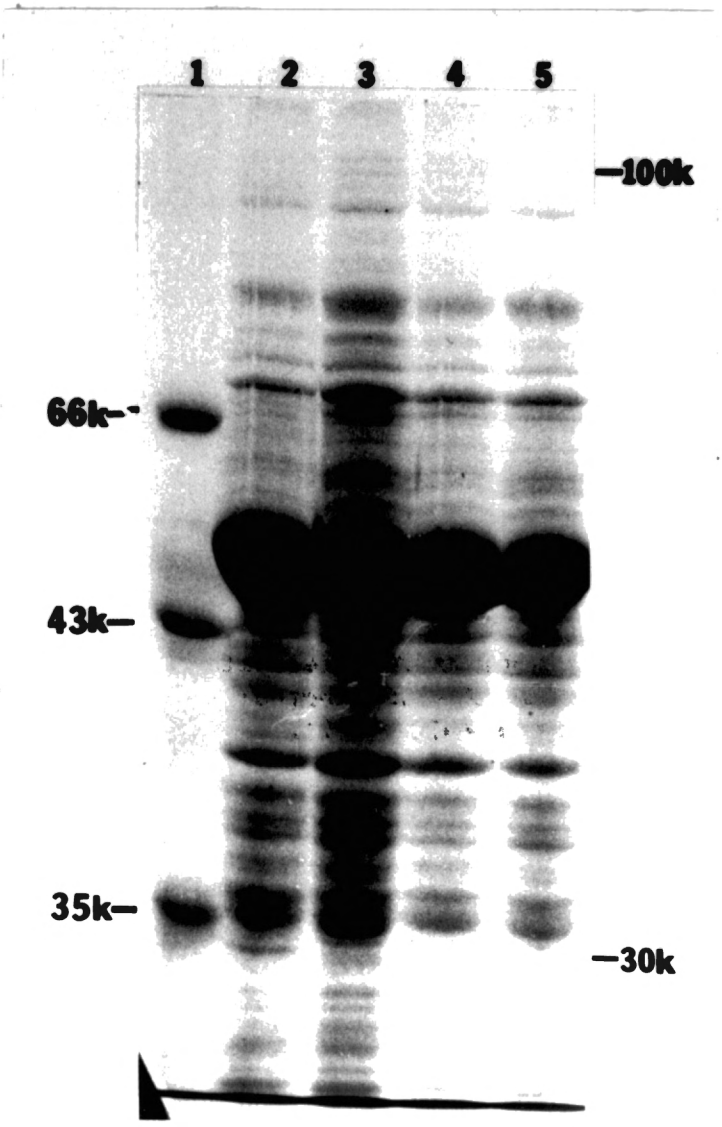

1
2
3
4
5
66k
43k
35k

Figure 13. Strain Comparisons of TCA Precipitates by SDS-PAGE. Lane 1 - standards; Lane 2 - uninfected; Lane 3 - CM4-184; Lane 4 - NY 8153; Lane 5 - CaB Davis.


1
2
3
4
5
100k
66k
43k
35k
30k

gel pattern is a band present in the supernatant sample of uninfected leaves but not of infected leaves that is no longer present in the infected samples. This polypeptide is located just below the 34K marker.

A number of proteins appear to be infection associated by comparison of the gel patterns from step three of the purification of inclusion bodies (Figure 14). These polypeptides have approximate M.W.'s of: 61,000; 56,000; 50,000; 43,000; 41,000; and 39,000.

Incubation of Extract

In an attempt to see if any of these polypeptides was degraded by proteases during the purification procedure, the total extract at step one of the purification was incubated at 37°C for two hours, after which the rest of the purification procedure was followed. The 61K polypeptide was no longer detectable by SDS-PAGE (Figure 15). The 56, 43, 41, and 39K polypeptides were still present, none of them increased in intensity. It appears that the 61K polypeptide was not converted to any of the other polypeptides. If the polypeptide were the inclusion body matrix protein then no inclusion bodies should be observed by EM. However, as can be seen in Figure 16, inclusion bodies are present, along with many small aggregates of what appears to be inclusion body material.

Figure 14. SDS-PAGE of Step Three of Purification Showing Polypeptides that are found with the Inclusion Body Fraction. Lane 1 - uninfected, Lane 2 - infected.

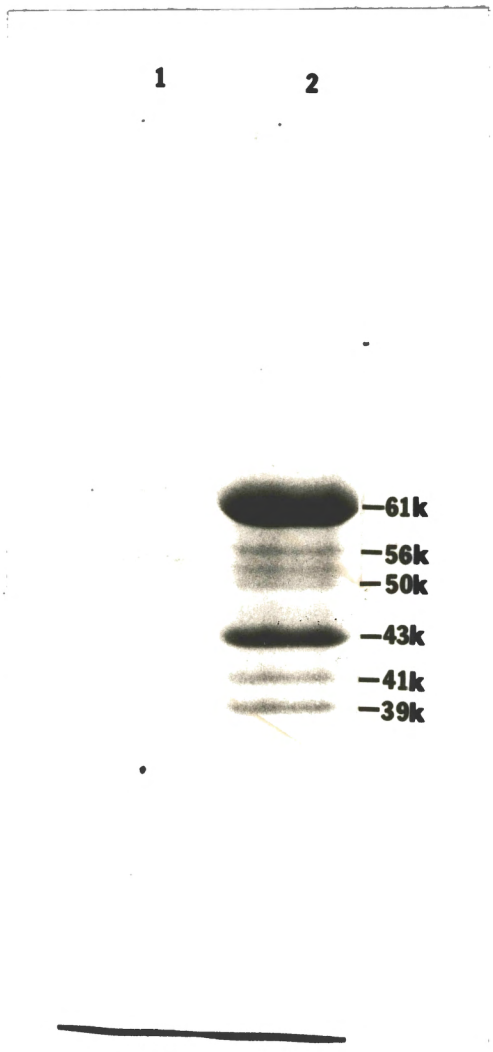

1
2
61k
56k
50k
43k
41k
39k

Figure 15. SDS-PAGE of the Inclusion Body Fractions Following Incubation (37°C for two hrs.) of the Total Extract. Lane 1 - uninfected; Lane 2 - nonincubated, infected, extract; Lane 3 - incubated, infected, extract; Lane 4 - standards.

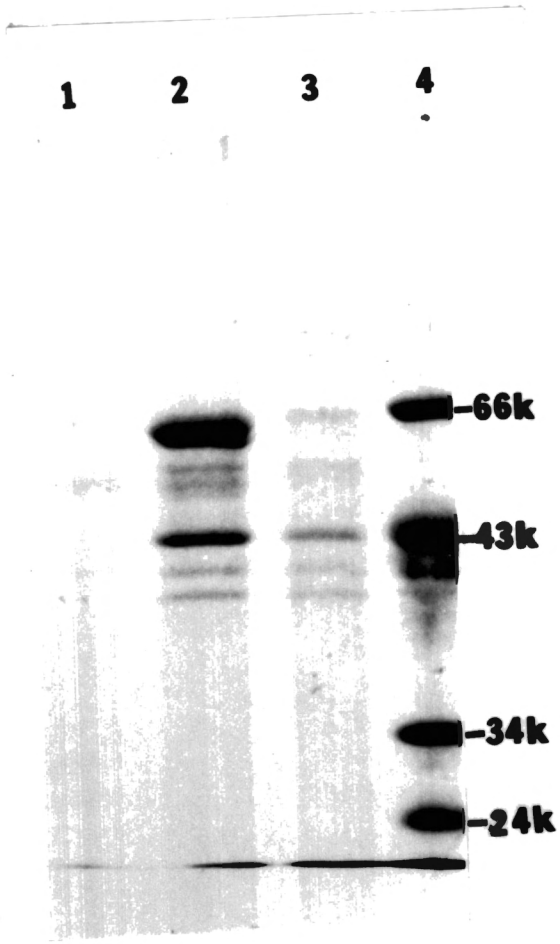
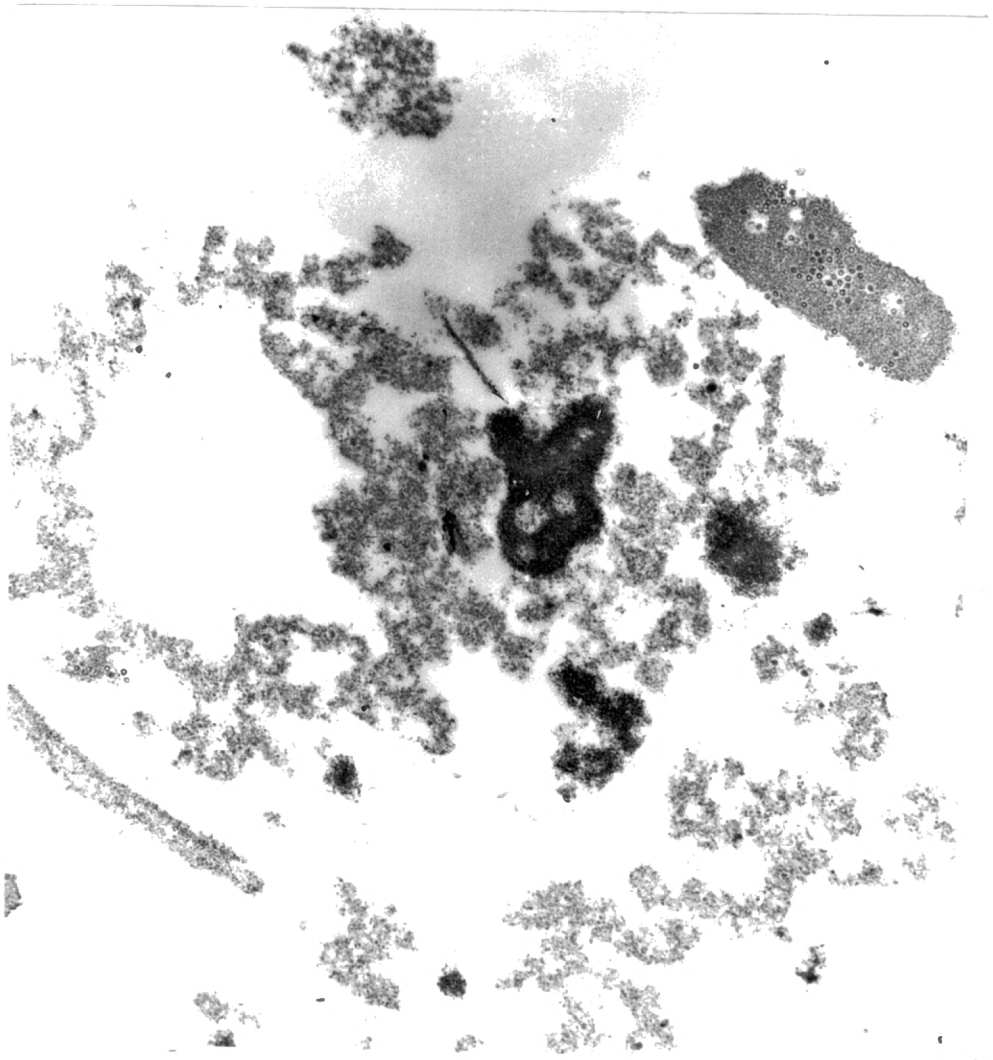

1
2
3
4
66k
43k
34k
24k

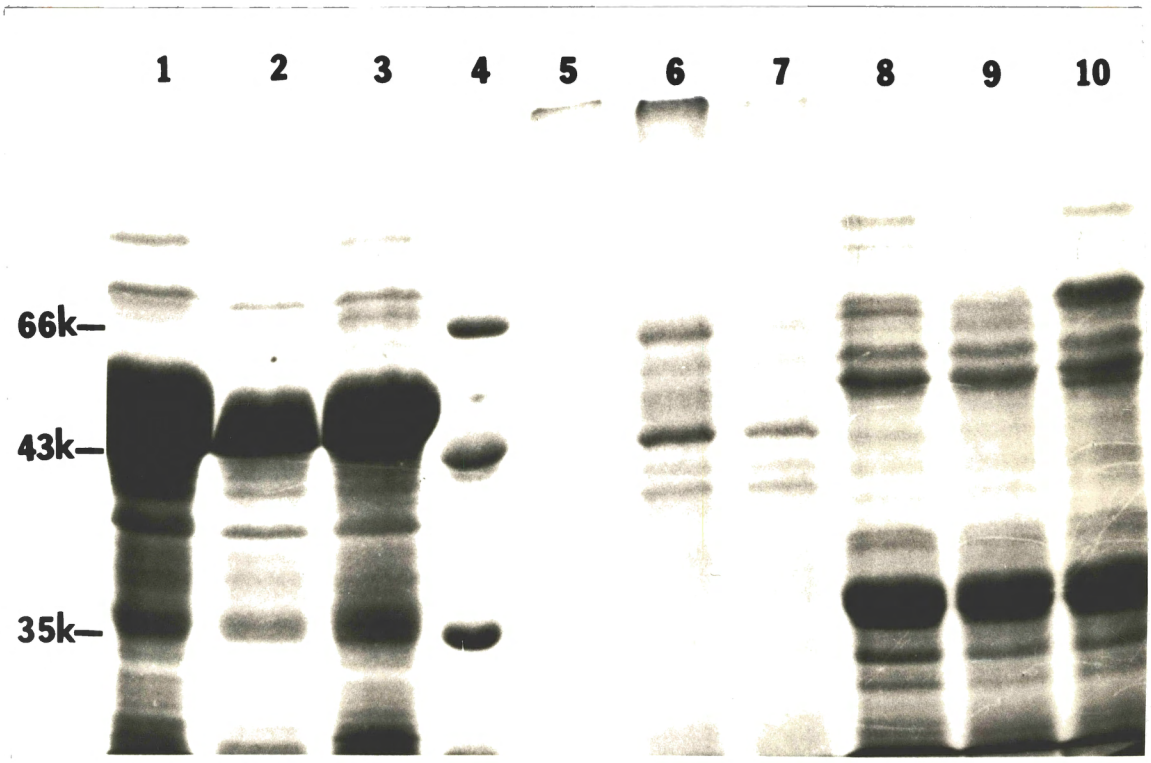
Figure 16. EM Photograph of Step Three of Inclusion Body Purification Following two hr. Incubation at 37°C. Intact Inclusion Bodies are still seen.



Comparison of Virus-associated Proteins in Infected Turnips (*Brassica rapa*, cv. Just Right) and Mustard (*Brassica perfiridis*, cv. Tendergreen)

To determine if proteins associated with infection are virus-coded or host-coded, both turnip and mustard were mechanically inoculated with CM4-184. The inclusion bodies were partially purified according to the purification procedure and their proteins were separated by SDS-PAGE. A 30,000 x g pellet was obtained by centrifuging the supernatant of step two of the purification and TCA precipitates were prepared from the supernatant of this centrifugation. The gel patterns for each of these three fractions are shown in Figure 17. All of the viral polypeptides associated with infected turnips at step three are also found in infected mustard. There is, however, one extra band found in the mustard just above the 61K polypeptide. The pattern of infected mustard and infected turnip polypeptides in both the 30,000 x g and TCA fractions are almost identical, probably reflecting the close phylogenetic relationship of these two species. Since polypeptides that do differ are also present or absent in uninfected turnips, these different polypeptides may be due to the difference in species.

Figure 17. Comparison of Polypeptides Isolated from Infected Turnips and Infected Mustard. Lanes 1, 2, and 3 - TCA ppts. of the supernatant of the 30,000 x g centrifugation. Lanes 5, 6, and 7 - inclusion body fraction. Lanes 8, 9, and 10 - pellet from step 2 supernatant centrifugation at 30,000 x g. Lanes 1, 5, and 8 - uninfected turnips, lanes 2, 6, and 9 - infected turnips, lanes 3, 7, and 10 - infected mustard, lane 4 - standards.


1
2
3
4
5
6
7
8
9
10
66k
43k
35k

CHAPTER V

SUMMARY AND CONCLUSIONS

The partial purification procedure for inclusion bodies that was adapted from Shepherd provided a method to examine the polypeptides associated with CaMV infection. Although purification was not complete, it will now be possible to attempt further purification, possibly using linear sucrose density gradients. Because some of the contaminating material may be ribosomes that are observed at the periphery and on the surface of the inclusion bodies by EM, it may be difficult to isolate inclusion bodies without these present.

The infection-associated polypeptides that have been found are: 100K, 68K, 61K, 56K, 50K, 43K, 41K and 39K. Two of these (61K and 43K) are the major polypeptides found in inclusion bodies. The 100K and 68K polypeptides are not found with the inclusion bodies. The 56K, 50K, 41K, 39K, along with the two major polypeptides, are found in conjunction with the inclusion bodies. If the polypeptides found in the inclusion body samples at step three of the purification procedure are compared with the viral polypeptides of Al Ani et. al. (1979), it appears that the 43K polypeptide corresponds to the major coat protein (P4). The 56K, 50K, 41K and 39K polypeptides correspond to their 55K, 49K, 39K and 37K polypeptides respectively. The relative intensities of the inclusion body bands supports their suggestion that the 43K polypeptide is the major polypeptide with the lower M.W.

polypeptides being degradation products of it. They stated that a freeze-thaw step might inactivate some proteases thus resulting in less degradation. Most CaMV purification procedures including Al Ani's require several days to carry out with overnight incubation with triton/urea. The procedure used here requires very little time - approximately six hours from time of removal of leaves until the sample is placed in dissolving buffer - which should preserve the major protein fairly intact. The presence of significant amounts of the lower M.W. polypeptides even during this short period may indicate that small amounts of these polypeptides are normally present in the virions.

Because it was of interest to know what possible differences in polypeptides there might be between different isolates of CaMV comparisons were made. It was hoped there would be a difference between CM4-184 (a non-aphid-transmissable isolate) and other aphid transmissable isolates. The only differences that could be seen by SDS-PAGE were the slightly slower mobilities of the 56K and 39K polypeptides in the CM4-184 sample. This may or may not be the difference that is being looked for.

This difference in mobility also supports the idea that these polypeptides are virus-coded. If they were host-coded, they should not migrate differently because the same host is being used. The 43K polypeptide is also probably virus-coded and it is likely that there is a difference in its mobility that cannot be observed in the SDS-PAGE patterns shown.

Shepherd and Wakeman (1977) indicated that the major inclusion protein had a M.W. of 55,000. The data here indicates that the M.W. of the most significant polypeptides present in partially purified

inclusion bodies is 61,000. It is possible that these polypeptides are the same, the differences in M.W. being due to inherent errors in determining M.W. of proteins on SDS-PAGE.

Odell and Howell (1978) reported the presence of a 66K polypeptide that sediments with a 30,000 x g centrifugation of the total extract. They stated that it was the major protein in leaf extracts of infected plants, and that it is not related to the virus coat proteins as shown by tryptic peptide analysis. They also stated that polysomes isolated from extracts could direct the synthesis of this polypeptide. This polypeptide may also be the same as the 61K polypeptide that is reported here, the difference in M.W. being due to errors in determining M.W. by SDS-PAGE. It has also been shown that the major polypeptides synthesized in a wheat germ system, using poly (A) containing RNA isolated from a low speed (500 x g) pellet, have M.W.'s of 61K and 43K (C. Gardner, personal communication). Since this fraction contains the inclusion bodies, it is likely that this mRNA is associated with the inclusion body rather than as soluble or membrane-bound polysomes.

The presence of a polypeptide in uninfected material that is not present in infected material may also be very important. This polypeptide (approximately 30,000 daltons) was detected only in uninfected material. It was not found in any of the three isolates of CaMV tested. It may be that supression of this polypeptide allows infection to proceed.

If one adds up the molecular weights of the polypeptides obtained in a partially purified preparation of inclusion bodies, there are approximately 290,000 daltons of protein present. The coding capacity of CaMV nucleic acid is approximately 350,000 daltons,

assuming no gene overlaps. Thus it appears that there is sufficient DNA to code for each of the peptides separately. If the 100K component found in the soluble fraction is added and the two supposed degradation products (41K and 39K) are subtracted, 310,000 daltons of coding capacity is required, which is still within the coding capacity of the nucleic acid.

A certain amount of information has been gained concerning the proteins associated with CaMV infection. There are however a considerable number of things that still need to be learned. Attempts to define the roles of the polypeptides have not been carried out and much research needs to be carried out to relate these polypeptides to their coding positions on the CaMV genone.

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